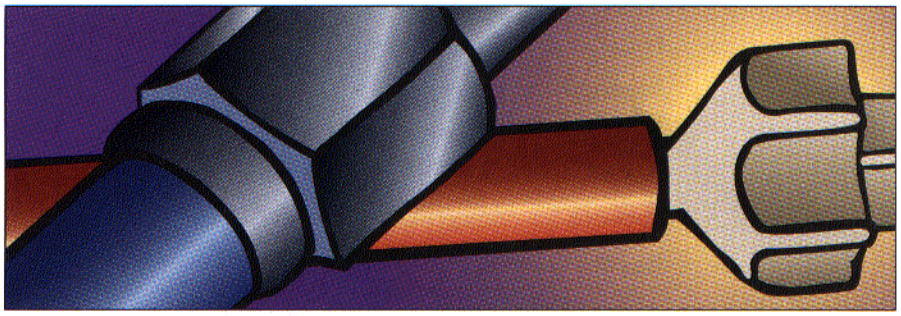
LC Troubleshooting



Air: Poison for Liquid Chromatographs

John W. Dolan, Daniel H. Marchand, and Shannon A. Cahill

Air is great when you need to breathe, but otherwise it is a source of many problems.

Perhaps the most commonly encountered liquid chromatography (LC) problem results from the presence of excess air in the mobile phase. Most of us are acutely aware of the pressure fluctuations and flow irregularities that happen when air gets into the pump. To avoid these consequences, most workers degas the mobile phase to help ensure reliable pump operation. Because air bubbles are so common, most of the problems are recognized readily and corrected with simple purging and degassing procedures. Air in the LC system can cause other problems that may be more subtle, and we will look at some of those problems in this month's "LC Troubleshooting."

THE PROBLEM

Not long ago, I received an e-mail that read something like the following:

We have used an LC system to assay both bulk drug and tablets. We used a 15 cm × 0.46 cm, 5-µm d_p C18 column and a guard column with a 40:40:20 (v/v/v) 60 mM phosphate buffer (pH 6.5)–methanol–acetonitrile mobile phase. The flow rate was 1.0 mL/min; the temperature was ambient; and the injection volume was 20 µL. We used UV-absorbance detection at 230 nm.

Using this system, we have seen — without exception — a peak at approximately 2.8 min, even when we injected only mobile phase. Before embarking on a series of experiments to establish what was causing the peak, we submitted the problem to our LC–MS group, who noticed that when the level of liquid in the vial was lower, the peak increased. Eventually, they showed that the peak was related to the amount of air in the injection solution. (When air alone was injected, the peak was huge.)

The retention of this peak has interfered with a potential degradant. We could degas the solutions before injection, but we need an explanation of what is going on. I would appreciate your thoughts about this problem.

John W. Dolan
LC Troubleshooting Editor

OUR EXPERIENCE

Your experience reminds me of a similar problem that we recently experienced in our laboratory. Our experimental setup was a bit different from yours. We used a 15 cm × 0.46 cm, 5-µm d_p C18 column with a 35:65 (v/v) 25 mM phosphate buffer (pH 2.5)–methanol mobile phase. The flow rate was 1.5 mL/min; the temperature was 30 °C; and the injection volume was 5 µL. We used UV-absorbance detection at 205 nm.

Our application was assaying a drug in plasma. For samples or spiked plasma, we normally saw chromatograms that looked similar to the one in Figure 1. Those samples received a solid-phase extraction cleanup, so the disturbance at the dead volume was minimal. In Figure 1, the expected dead time (t_0) upset is at 1 min.

It is good to keep an eye on the dead time, because it provides a running check on the flow rate. You can estimate the column volume for 4.6-mm i.d. columns as $0.1L$, where L is the column length in centimeters, and the volume is in milliliters. Thus for a 15-cm column, the volume is approximately 1.5 mL. The column dead volume is converted to the dead time by dividing it by the flow rate. For the chromatogram of Figure 1, $t_0 \approx 1.5/1.5 = 1.0$ min. This estimate of t_0 should be good to approximately 10%. The repeatability of t_0, however, should be better — as good as the combination of the repeatability of the autosampler timing and the pump delivery, which is better than 2% for most systems. If you see the t_0 disturbance moving from run to run, it is an indication that either the pump or the autosampler is working improperly.

In addition to observing a highly reproducible dead time peak, we saw our analyte's peak eluted at 2.24 ± 0.05 min. This gave a retention factor (k) of approximately 1.25, which was satisfactory for a method with no significant peaks at the column dead volume. A couple of minor plasma components were eluted after the sample peak at 2.64 and 3.13 min, but they were well resolved from the analyte. The chromatogram in Figure 1 was typical for the method and provided quantitative results for our application.

One morning we examined the data from the previous night's runs and encountered chromatograms similar to the one in Figure 2. A broad peak appeared at approximately 2.4 min — and as you can see in Figure 2 — it interfered with the quantitation of our analyte. We set about isolating the source of the problem.

Late-eluted peak? With LC separations, the peak widths for all the peaks in a given region of the chromatogram should be approximately the same. For gradient separations, the peak widths tend to be the same throughout the run. For isocratic methods, the peaks get broader

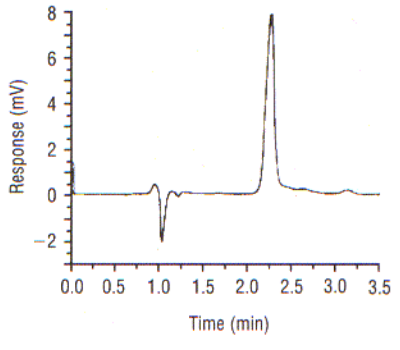


FIGURE 1: Normal chromatogram showing the analyte peak at 2.24 min. See text for details.

the longer the analytes are retained. In the case of Figure 2, the unwanted peak was considerably broader than the analyte peak, so our first assumption was that it was retained from a previous injection.

We tested this hypothesis simply by reinjecting the sample. If the run time is extended, the peak should be eluted eventually. When we reinjected the sample, the chromatogram looked very similar to the one in Figure 2, although the relative size of the two peaks had changed. The constant retention time of the problem peak told us that it was not a late-eluted analyte but truly was retained at 2.4 min.

Metabolite? Once we eliminated the late-eluted peak from contention, our next suspect was a new metabolite. When assaying drugs in plasma, it is not unusual to find individual differences in metabolism. In combination with coadministered drugs, this effect can result in interfering peaks in some samples that do not appear with otherwise identical samples.

When you suspect that metabolites are the source of a problem, you should change the chromatographic conditions to try to separate the peaks, just as for any other chromatographic peak pair. Tracking down a metabolite problem can be a big job, so before we embarked on this route, we decided to reinject a few more samples to see if we could characterize the problem a little better. In each case, we saw the 2.4-min peak, even when we reinjected samples that earlier had shown no interference. We began to suspect sample degradation but reinjection of freshly prepared samples still showed the interference at 2.4 min. We injected a mobile-phase blank, and the 2.4-min peak persisted.

Autosampler? When interference appears in every sample — including blank injections — the presence of a contaminant is likely. Because the peak was common to all samples and blanks, we could eliminate the sample preparation technique as a source of contamination. The autosampler was a logical source of contamination. If the wash solution had become contaminated or some other source of contamination existed, a chromatographer could inadvertently introduce the unwanted peak in every sample.

When we started to examine the autosampler, we noticed that the amount of sample remaining in the sample vials varied from vial to vial even though they all contained the same volume initially. Was it possible that the autosampler was injecting only part of the sample? Our autosampler can be programmed to adjust the needle depth in the vials. When we checked this adjustment, we discovered that the injection needle was not reaching the bottom of the vial.

Could it be air? Immediately, we suspected that the problem peak was related to air being injected with the sample. Figure 3 shows the results from an intentional injection of 5-µL of air.

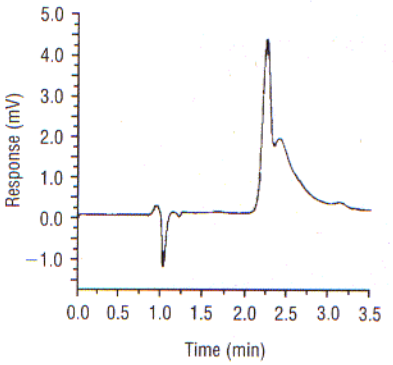


FIGURE 2: Chromatogram showing the analyte peak plus an interfering peak eluted at 2.4 min. See text for details.

The peak *was* air.

We reprogrammed the autosampler to ensure that the needle was inserted to the correct depth in the vials. After we made this adjustment the system performance returned to normal, and we once again obtained chromatograms similar to Figure 1.

Other possibilities: We encountered a similar air-injection problem once before when we allowed the autosampler wash reservoir to run dry. Although the wash solvent normally is not injected with the sample, all the tubing between the injection syringe and the sample needle filled with air when the reservoir was dry. Now, instead of having a precise, hydraulic link between the syringe and the needle, we had established a readily compressible air link. The compressibility of air in the tubing caused poor injection precision, and, at times, air was injected with the sample. Refilling the reservoir and purging the connecting tubing solved the problem in that case.

Some detectors are more sensitive to air than others. Air is particularly problematic with UV detection in the 195–230 nm region. Electrochemical detection in the reductive mode also is very sensitive to oxygen in the mobile phase. This problem is big enough that chromatographers may need to replace all polytetrafluoroethylene (PTFE) tubing in the system with stainless steel or polyetheretherketone (PEEK) tubing. PTFE is quite permeable to air, and enough air can diffuse through the tubing to cause detection problems in the reductive mode.

CONCLUSIONS

You can see that air can produce peaks that look surprisingly like normal chromatographic peaks, and that air can be significantly and reproducibly retained. The best way to avoid injection of air is to make sure that the sample vial contains sufficient sample volume for the method requirements and that the needle is inserted to an adequate depth. In our laboratory, we were able to enhance autosampler reproducibility by degassing the autosampler wash solvent and purging the autosampler with freshly degassed solvent on a daily basis.

In some cases, you may need to degas the samples to remove the last traces of air — as did the reader who submitted this month's question.

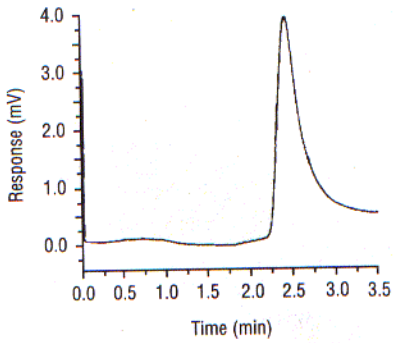


FIGURE 3: Chromatogram obtained from the injection of 5 µL of air. See text for details.

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ERRATUM

In February's "LC Troubleshooting" (*LC•GC* **15**[2], 112 [1997]), the sidebar "Two Simple Tests" contains an error in the last sentence. The pump specifications should read 1% and 2%, not 0.1 % and 0.2%.